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TITLE: Control of Venous Thromboembolism and Metastasis in Breast Cancer by Anti-Fibrinolysis Mechanism

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<b>13. SUPPLEMENTARY NOTES</b>					
<b>14. ABSTRACT</b> Formation of abnormal clots in vessels is a life-threatening situation and the second leading cause of death in cancer patients and is particularly common in breast cancer. Clot formation may also enhance tumor cells spread throughout the body. This study shows a tumor-related cell surface enzyme FAP as a potential contributor to abnormal clot formation and tumor cell survival and growth in blood mimics. Based on its results, new tests can be developed to identify breast cancer patients at high risk of developing abnormal clots. These patients can benefit most from preventive treatments. Also, agents specifically inhibiting the above enzyme have been investigated in this project to evaluate their use in preventing abnormal clot formation.					
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## Introduction

Recently, fibroblast activation protein (FAP)/seprase was shown to exhibit a novel function by cleaving an N-terminal peptide of  $\alpha_2$ -antiplasmin to promote the binding to its target, plasmin, increasing inhibition of fibrinolysis, and maintaining clots formed in vessels (1, 2). FAP is a peculiar serine protease with prolyl peptidase and gelatinase activity that was originally described in “activated” normal and tumor cells in certain human malignant tumors including infiltrating ductal carcinomas of the breast and its metastases (3-9). In addition, literature search shows that metastatic tumor growth is associated with local fibrin deposition and clot formation in the circulation (10-14). While the physiologic role of FAP and its substrate(s) have remained mostly elusive, there is no reports to date, indicating the role of FAP in the circulation of breast cancer patients. Investigation into this subject is significant for breast cancer treatment, as FAP may play an important role in the survival of metastatic tumor cells in the circulation (15) and the increased risk of venous thromboembolism (VTE) in vital organs such as the lung and brain in breast cancer patients. The award led to the demonstration that FAP produced by tumor cells in culture inhibits fibrinolysis on the cell surface that potentiates tumor cell survival and maintain clot in human blood mimics.

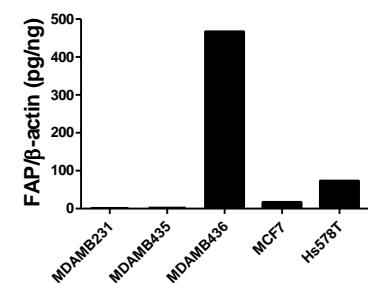
## Body

[Please note that the term “blood” in the approved Statement of Work has been changed to “blood mimics”. This is because the grant involves in vitro experiments using tumor cell lines and use of animal and human blood is not allowed.]

### Task 1:

**To characterize FAP expression profile in various breast tumor cell lines and generate FAP-knockdown variants.** We have completed the FAP expression profiling experiments, and found that only Hs578T and MDA-MB-436 human breast carcinoma cell lines expressed high levels of FAP RNAs but not MDA-MB-435 (a discontinued breast tumor cell line from the ATCC due to general concerns on over-expression of melanoma genes in this cell line; data were generated using a line stored in liquid nitrogen in our lab over 10 years), MCF7 and MDA-MB-231 human breast carcinoma cell lines (Figure 1). However, FAP protein expression by Western

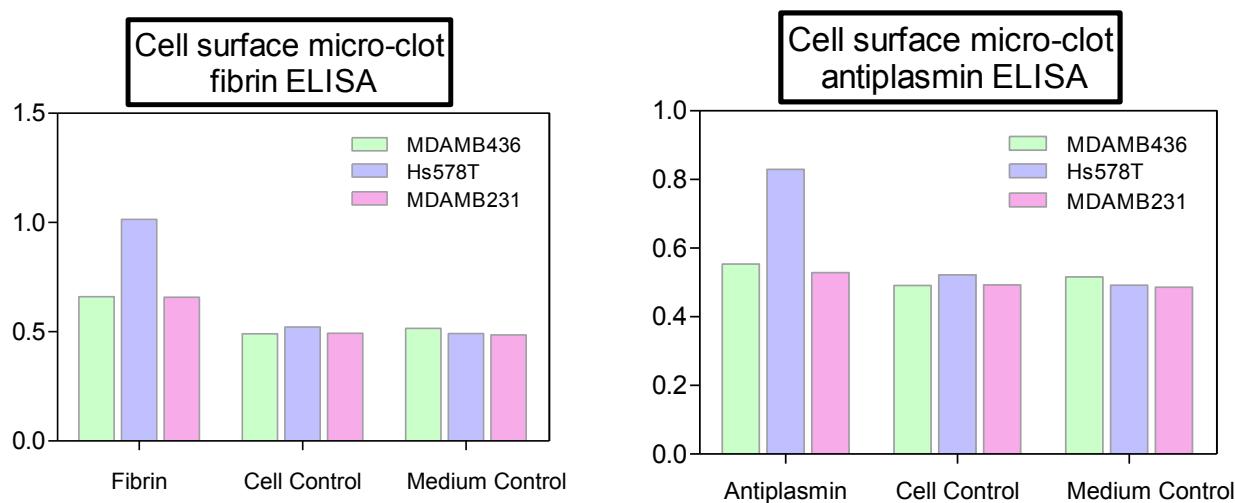
immunoblotting of cell lysate was not correlated with quantitative ELISA and the FAP-associated proteolytic activity of intact and viable cells derived from Hs578T and MDA-MB-436 lines. This suggests that, in MDA-MB-436 cells, the majority of FAP protein is distributed inside the cell, and there is unlikely to have FAP function on the surface of these cells. The knockdown approach originally planned is, therefore, unlikely to generate cell lines that address the question. Thus, the proposed knockdown experiment was not performed.



**Figure 1. FAP expression profiling of breast carcinoma cell lines revealed using qPCR.**

**Task 2:**

**To examine the ability of FAP<sup>+</sup> and FAP<sup>-</sup> breast tumor cells to inhibit fibrinolysis and maintain micro-clot formation around breast tumor cells.** Using ELISA to determine cell surface micro-clot, we have found that the surface of Hs578T FAP<sup>+</sup> human breast carcinoma cells are covered with micro-clot but not MDA-MB-436 FAP<sup>+</sup> human breast carcinoma cells and MDA-MB-231 FAP<sup>-</sup> human breast carcinoma cells (Figure 2). Consistently, using immunocytochemical localization assay, concentration of fibrin and antiplasmin, downstream substrates of the active cell surface FAP enzyme, is higher on the cell surface of Hs578T FAP<sup>+</sup> human breast carcinoma cells but lower on MDA-MB-436 FAP<sup>+</sup> human breast carcinoma cells and MDA-MB-231 FAP<sup>-</sup> human breast carcinoma cells. The data supports our original proposal that membrane-bound FAP is capable of protecting the micro-clot formed around tumor cells from fibrinolysis.



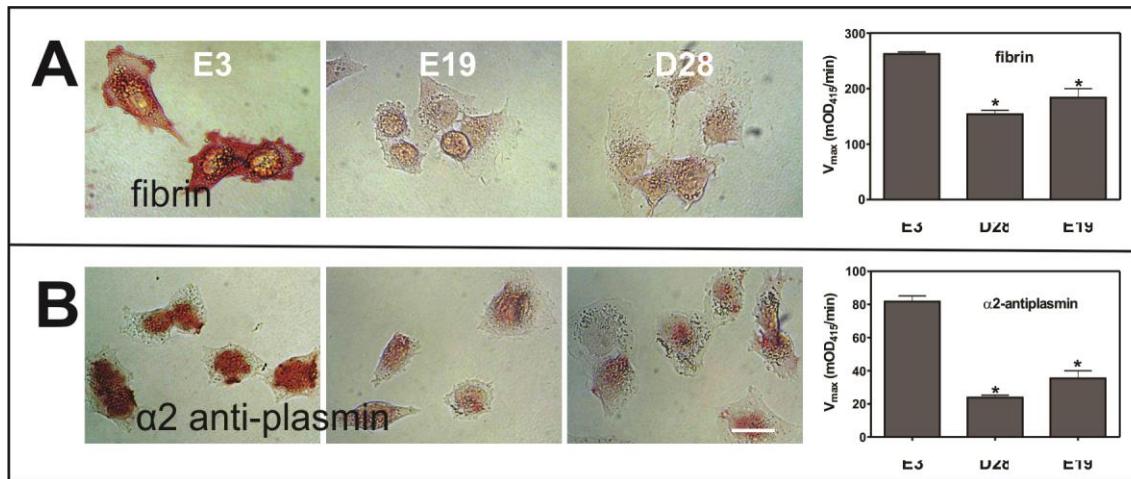
**Figure 2. Cell surface micro-clot formed on breast carcinoma cell lines revealed using fibrin and antiplasmin ELISA.** 1% dilution of de-identified human plasma was prepared in serum-free culture medium. Tumor cells were added to plasma-containing medium at 25,000 cells/mL and 200  $\mu$ L of tumor cell suspension (5,000 cells per well) was added to each well in a 96-well microtiter plate. Wells were incubated overnight; cells were fixed with 3.5% paraformaldehyde/PBS; and stained with antibodies against fibrin or antiplasmin, followed by secondary antibodies and color reaction. Controls included cell layers (Cell Control) or medium conditioned by respective cell types (Medium Control) proceeded similarly except in the absence of primary antibodies against fibrin or antiplasmin. Measurements were made among breast carcinoma cells indicated in terms of the amount of fibrin or antiplasmin formed around the cell surface of each cell line.

**Task 3:**

**To examine the ability of FAP<sup>+</sup> and FAP<sup>-</sup> breast tumor cells to survive and grow in blood mimics.** The role of FAP in survival and growth of tumor cells in the circulation has been investigated *in vitro* by examination of FAP<sup>+</sup> and FAP<sup>-</sup> breast tumor cells cultured in plasma for 8 days. We found that proliferation rates of Hs578T FAP<sup>+</sup> cells were twice higher than that of FAP<sup>+</sup> (intracellular) MDA-MB-436 and FAP<sup>-</sup> MDA-MB-231 FAP<sup>-</sup> tumor cells, similar to the FAP expression on the cell surface of these tumor cells (see also Figure 1 above).

**Task 4:**

*To test if FAP catalytic inhibitors can inhibit its putative role in fibrinolysis that, in turn, prevent the micro-clot formation on tumor cell surfaces, and inhibit cell survival or growth in blood mimics.* This experiment has been accomplished by examination of the SB247A tumor cells cultured in 1% plasma (as optimized from Tasks 2 & 3) in the presence of anti-FAP and DPP4 mAb inhibitors for 8 days. Specifically, 5,000 cells per well, the anti-FAP mAb D28, anti-DPP4 mAb E19, and control anti-DPP4 mAb (10 µg/mL each), and de-identified human plasma samples were added into a 96-well plate to media containing plasma. Comparisons have been made among experimental and control groups in terms of the “micro-clot” formation on tumor cell surfaces, and cell growth (Figure 3).



**Figure 3. Demonstration of the hypothesis that FAP catalytic inhibitors can inhibit its role in fibrinolysis that, in turn, prevent the micro-clot formation on tumor cell surfaces (left images), and inhibit cell growth (right plots).** The SB247A tumor cells were cultured in 1% plasma in the presence of anti-FAP and DPP4 mAb inhibitors for 8 days. Specifically, 5,000 cells per well, the anti-FAP mAb D28, anti-DPP4 mAb E19, and control anti-DPP4 mAb (10 µg/mL each) developed in our laboratory, and de-identified human plasma samples were added into a 96-well plate to media containing plasma. Comparisons have been made among experimental and control groups in terms of the “micro-clot” formation on tumor cell surfaces and cell growth. Bar=40µm.

## Key Research Accomplishments

- 1) Characterize FAP expression profile in a panel tumor cell lines and determine a tumor cell line that expresses functional FAP on the cell surface. This is important as our original proposal using FAP-knockdown could not obtain the tumor cell line expressing “functional FAP” on the surface.
- 2) Demonstrate by fibrin and antiplasmin ELISA that the ability of FAP+ and FAP- breast tumor cells to exhibit the cell surface fibrinolysis and maintain micro-clot formation around breast tumor cells.

- 3) Demonstrate by cellular proliferation rates in culture that FAP+ tumor cells grew twice faster than FAP- tumor cells in blood mimics.
- 4) Demonstration by anti-FAP neutralizing antibodies that FAP catalytic inhibitors can inhibit its role in fibrinolysis that, in turn, prevent the micro-clot formation on tumor cell surfaces (Figure 3, left images) and inhibit cell growth (Figure 3, right plots).

## Reportable Outcomes

Mr. Mazyar Javidroozi obtained a PhD in 2010 that was supported by this award.

## Conclusion

In conclusion, the award supports us to demonstrate the hypothesis that FAP catalytic inhibitors can inhibit its role in fibrinolysis that, in turn, prevent the micro-clot formation on tumor cell surfaces, and inhibit cell growth in blood mimics. The concept is novel and has significant potential implications in care of breast cancer patients. Moreover, the proposed approach to investigate the role of FAP in maintenance of “micro-clot” coat on tumor cell surfaces is highly innovative. Further translational research may lead to the development of a novel test to identify breast cancer patients at increased risk of VTE who can benefit most from anticoagulative prophylaxis, as well as use of FAP catalytic inhibitors as prophylactic/therapeutic agents to prevent VTE and metastatic progression in breast cancer patients. The results from this project enable further investigation to validate these results in animal models as well as in cancer patients. More than five years of continued investigation will likely be needed before the commercial development of potential drugs to prevent/treat abnormal clots and tumor spread in breast cancer patients. The results of this study could have an enormous impact on the survival and quality of life of breast cancer patients.

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## Appendices

N/A.